Low energy nanoemulsions as a carrier of thyme and lemon balm essential oils

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Abstract

Essential oils (EOs) have been suggested as an alternative to synthetic preservatives and, despite their instability, their encapsulation in nanoemulsions (NEs) could promote their incorporation in foods. Thus, this work aims to develop nanoemulsions by a low energy method for the encapsulation of thyme and lemon balm EOs. Nanoemulsions were prepared by the emulsion phase inversion method with sunflower oil as the carrier oil, and different surfactant-to-oil ratios (SOR) and EOs loading were evaluated. The physical stability, antimicrobial activity, cytotoxicity and antioxidant properties of the NEs were determined. Nanoemulsions presented a monomodal size distribution below 200 nm and a high negative zeta potential (> -40 mV). NEs stored at 4 °C without EOs were stable during 6 months and nanoemulsions with EOs and SOR 2 for 3 months. Nanoemulsions with EOs presented antimicrobial activity against *S. aureus* and cytotoxicity in Caco-2 cells when above 100 μg/mL at 48 hours of exposure.

Keywords: emulsions; emulsification; nanotechnology; encapsulation, bioactive compounds.

Abbreviations: NE – nanoemulsion; EO – essential oil; EPI – emulsion phase inversion method; SE – spontaneous emulsification; PIT – phase inversion temperature; MCT – medium chain triglycerides; LCT – long chain triglycerides; DLS – dynamic light scattering; PDI – polydispersity index; TEM – transmission electron microscopy; SOR – surfactant-to-oil ratio; PCA – Plate Count Agar.

1. Introduction

Food related diseases are growing worldwide and new efficient strategies for its reduction are needed (Granata et al., 2018). Food additives may represent a threat for human health and, despite their bioactivities, other effects may arise. Currently, the use of synthetic antimicrobials and antioxidants is being avoided, due to their limited activity against some pathogenic microorganisms, and due to their toxicity, carcinogenic effects and potential environmental risk (Das et al., 2019; Zhang, Vriesekoop, Yuan, & Liang, 2014). Regulatory agencies and companies are promoting the use of natural compounds; moreover, consumers are currently more concerned about sustainability and their health and, therefore, the use of natural products is seen as a way to avoid the use of synthetic ones (Acevedo-Fani, Soliva-Fortuny, & Martín-Belloso, 2017; Granata et al., 2018).

Essential oils are globally considered potential antimicrobial agents for food preservation, as they have antifungal and antibacterial properties against a wide range of pathogenic agents transmitted by food and/or microorganisms responsible for food spoilage. Additionally, they have important antioxidant, anti-viral and insecticidal activities (Pandey, Kumar, Singh, Tripathi, & Bajpai, 2017). Thus, essential oils represent more ecologic and safe alternatives to the treatment of infectious diseases, also contributing to improve the quality and shelf-life of food products (Pandey et al., 2017). The European Union Commission approved the use of essential oils in foods on the Regulation (EC) No 1334/2008 (European Commission, 2008). Likewise, the Food and Drug Administration recognises essential oils as safe substances (GRAS - Generally Recognised as Safe) (Pandey et al., 2017; U. S. Food and Drug Administration, 2019).

Thyme and lemon balm, common names for *Thymus vulgaris* L. and *Melissa officinalis* L., respectively, are aromatic perennial subshrubs from the family Lamiaceae. They had origin in the South of Europe and in the Mediterranean region and are both chemically variable species (Avci & Giachino, 2016; Nabavi et al., 2015). Thyme is traditionally used as a culinary ingredient. In folk medicine, it has been used as expectorant, diuretic, antispasmodic, carminative and anti-smoking agent, and in the treatment of laryngitis, bronchitis, cough, urinary tract infections and gastrointestinal disorders (Borugă et al., 2014; Nabavi et al., 2015). Lemon balm is used since ancient times in culinary, perfumery and cosmetics (Turhan, 2006). In traditional medicine, lemon balm has been used to treat several conditions and diseases like depression, anxiety, insomnia, headaches, bronchitis, asthma, toothache, indigestion, hypertension, fever and acne (Turhan, 2006). Essential oils from these two plants have antibacterial, antifungal and antioxidant properties (Avci & Giachino, 2016; Nabavi et al., 2015). Thyme essential oil is also anti-inflammatory (Nabavi et al., 2015) and lemon balm oil has anti-viral (Avci & Giachino, 2016) and anti-tumoral activities (Sousa et al., 2004). The minimum amount of essential oil extracted in thyme is 12 mL/kg (Borugă et al., 2014), while the total content of essential oil that can be found in lemon balm range between 0.1 and 3.0 mL/kg. This small amount leads to the increase of lemon balm production cost and therefore rising the commercial value of its essential oil (Avci & Giachino, 2016; Turhan, 2006).

However, the use of essential oils in foods has several limitations, since they are commonly unstable and oxidise easily, being sensitive to some physicochemical factors, such as high temperatures, light and pH, that difficult their incorporation into food products (Granata et al., 2018). Moreover, their use in some foods and beverages is sometimes limited due to their low solubility in water (Moraes-Lovison et al., 2017), strong flavour/odour, which can alter the organoleptic characteristics of the product (Guerra-Rosas, Morales-Castro, Ochoa-Martínez, Salvia-Trujillo, & Martín-Belloso, 2016; Moraes-Lovison et al., 2017), and due to their potential toxicity at high doses (Acevedo-Fani et al., 2017).

In order to overcome these limitations, it is crucial to develop methodologies that preserve the

components of essential oils from undergoing reactions that compromise the effectiveness of their bioactivity. The use of nanoemulsions can be a successful approach, since they are able to improve the physical and thermal stability of active ingredients, as well as their solubility, absorption, bioavailability and also allow their control release (Granata et al., 2018). Similar to what happens for other hydrophobic compounds, the encapsulation of essential oils in nanoemulsions, usually increases their antimicrobial activity (Li, Zhang, Yuan, Liang, & Vriesekoop, 2013; Moraes-Lovison et al., 2017), reducing the concentration needed and consequently the risk of toxicity caused by high doses (Acevedo-Fani et al., 2017). Moreover, nanoemulsions have greater long-term stability than conventional emulsions and moderated optical clarity, which are important characteristics for several applications into food and beverage products (Li et al., 2013).

Nanoemulsions are a class of emulsions, with a diameter at the nanoscale, being one of the nanoencapsulation systems most explored in the food industry. They usually present diameters between 20-200 nm and, depending on its droplet size, they can be transparent or milky white (Acevedo-Fani et al., 2017; Borrin, Georges, Moraes, & Pinho, 2016). Due to their small particle size, nanoemulsions are more stable to gravitational separation, coalescence and flocculation than the conventional emulsions, but they are susceptible to Ostwald ripening (Chang, McLandsborough, & McClements, 2015). Besides they can improve the bioavailability of the bioactive compounds due to their small droplet size and high surface area (Acevedo-Fani et al., 2017; Chang & McClements, 2014; Silva, Cerqueira, & Vicente, 2011).

There are two different approaches to produce nanoemulsions: high and low energy methods. High energy methods rely on equipment that applies high energy to disturb and blend the oil and water phases, leading to the formation of small droplets (Silva, Cerqueira, & Vicente, 2015). Low energy methods are based on the spontaneous production of small droplets when the system composition or environmental conditions are changed, taking advantage of the chemical characteristics of the components used in the formulation (Chuesiang, Siripatrawan, Sanguandeekul, McLandsborough, & Julian McClements, 2018; Komaiko & McClements, 2015; Silva et al., 2011).

105 Low energy methods have several advantages: simple equipment required, low operating costs and
106 less energy needed being more energy-efficient (Chuesiang et al., 2018; Li et al., 2013; Mayer, Weiss,
107 & McClements, 2013). Moreover, as these methods do not use high energy, the degradation of
108 thermolabile active agents during the encapsulation process is avoided (Silva et al., 2011).

109 In the present work, nanoemulsions were produced through the emulsion phase inversion (EPI)
110 method, due to the sensibility of essential oils to high temperatures. Moreover, according to the
111 literature, this method leads to smaller particles than other low energy methods (spontaneous
112 emulsification) in similar systems (Komaiko & McClements, 2015). The EPI method is also simple
113 and easy to perform, that relies on a catastrophic phase inversion that occurs with the titration of the
114 aqueous phase over the organic phase, which is composed by oil and surfactant, under continuous
115 agitation (Borrin et al., 2016). Most of the published works with encapsulation of essential oils into
116 nanoemulsions are focused on high energy techniques and only a few explore the use of low energy
117 techniques. Two recent works reported the production of thyme EO-loaded nanoemulsions using
118 spontaneous emulsification (SE) and emulsion phase inversion (EPI) methods (Miastkowska,
119 Michalczyk, Figacz, & Sikora, 2020; Ryu, McClements, Corradini, & McLandsborough, 2018). In
120 those works, nanoemulsions had small sizes (approximately 50 nm, when the SE method was used,
121 and 15 nm when the EPI method was used), however, the polydispersity index of these formulations
122 was not satisfactory (between 0.2 and 0.3 for the SE method and 0.4 for the EPI method), and
123 polymodal size distributions were obtained. Regarding, low-energy nanoemulsions loaded with
124 lemon balm essential oil, and to best of authors' knowledge, there is no published work on their
125 production and characterisation. It is also scarce the full study of the active properties (antioxidant
126 and antimicrobial) of loaded nanoemulsions and their possible cytotoxicity. The main aim of this
127 study was the production and characterisation of nanoemulsions loaded with thyme or lemon balm
128 essential oils through the EPI method. The stability over time in different storage conditions, the
129 antimicrobial and antioxidant activities and cytotoxicity were assessed.
130

2. Materials and Methods

2.1. Materials

Refined sunflower oil (3ás, Fula, Sovena Group, Algés, Portugal), Tween 80 (P1754, Sigma-Aldrich, St. Louis, Missouri, USA) and ultrapure water (Milli-Q, Darmstadt, Germany) were used for the nanoemulsions production. Pure oils of thyme (*Thymus vulgaris*) and lemon balm (*Melissa officinalis*) were kindly supplied by the company Earth Essences (Póvoa de Lanhoso, Portugal). TEM grids (ultra-thin carbon film on Lacey carbon support film, 400 mesh, Copper, ref. 01824) were acquired from Ted Pella Inc. (Redding, California, USA) and UranyLess (22409) from Electron Microscopy Sciences, Hatfield, Pennsylvania, USA. DPPH (D9132), Trolox (23881), ABTS (A1888) and Plate Count Agar (PCA) plates were provided from Sigma-Aldrich, St. Louis, Missouri, USA. Ethanol 99% was purchased from Honeywell (Charlotte, North Carolina, USA), and 96-well microplates (611F96) were acquired from Thermo-Fisher (Waltham, Massachusetts, USA).

2.2. Nanoemulsions preparation

Nanoemulsions were produced by the Emulsion Phase Inversion (EPI) method as presented by Ostertag et al. (2012), with some modifications. The organic phase was prepared by mixing the surfactant Tween 80 and the oil (10 wt %), or a mixture of oil and EOs, at 750 rpm for 30 minutes. Ultrapure water was used as the aqueous phase. The titration of the water into the organic phase was made with a syringe pump (NE- 1000, New Era Pump Systems, Farmingdale, New York, USA) at a flow rate of 4 mL/min under agitation (750 rpm) for 60 minutes. Different surfactant-to-oil ratios (SOR) were tested, based on preliminary studies; 1.0, 1.5 and 2.0. The agitation was performed using an overhead stirrer VOS 14 S40 (VWR, Radnor, Pennsylvania, USA) equipped with a metal 4-blade tool. Samples were named as NE_X_YZ, with X being the SOR, Y indicates if thyme (T) or lemon balm (LB) essential oils were used, and Z is the wt% of essential oil in the formulation.

2.3. Particle size and Zeta potential measurement

Particle size, polydispersity index (PDI) and zeta potential measurements were performed using a dynamic light scattering (DLS) with a detection angle of 90° and 173°, respectively (Horiba SZ-100, Quioto, Japan) at 25 °C. Before analysis, samples were diluted 500x in Milli-Q water (Komaiko & McClements, 2014). For size distribution and PDI determinations polystyrene cuvettes were used and for the zeta potential measurement an electrode cell of carbon with 6 mm was used.

2.4. Transmission electron microscopy (TEM)

Nanoemulsions were negatively stained with UranyLess on TEM grids after dilution with Milli-Q water (10x). Samples were observed using a JEM-2100 transmission electron microscope (JEOL, Akishima, Japan) operating at 200 kV accelerating voltage.

2.5. Stability study

The physical stability of formulations over time was assessed by both DLS analysis (as described in 2.3) and visual inspection, at two temperatures: 20 and 4 °C. Samples without essential oil were followed during 6 months, while samples with essential oils were monitored during 3 months, whenever possible.

2.6. Antimicrobial activity

The antibacterial activity was tested against two bacterial strains: *Staphylococcus aureus* CECT 240 (Gram-positive) and *Escherichia coli* CECT 516 (Gram-negative) (Spanish Type Culture Collection, Valencia, Spain) by the disc agar diffusion test. Plate Count Agar (PCA) was prepared based on the supplier instructions. The bacteria culture was grown in Nutrient broth medium at 37 °C during 24 h and 0.1 mL was inoculated in PCA plates. Sterile paper discs were immersed in 50 µL of different nanoemulsion solutions and placed on the surface of each inoculated plate. The agar plates were incubated for 24 h at 37 °C and diameters of the inhibitory zone of clearance (cm) surrounding

the discs were measured to estimate the antimicrobial activity. Samples without essential oil and sterile paper discs were used as controls.

2.7. Antioxidant activity

2.7.1. DPPH assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging test was carried out using the method described by Ballesteros et al. (2015), with some modifications. 150 μM DPPH and a stock solution of 1000 μM Trolox were prepared in ethanol 99%. A calibration curve was prepared using different Trolox concentrations. Samples were diluted in ethanol 99% (1:20). The DPPH solution was dissolved in ethanol to an absorbance value of 0.70 ± 0.02 at 515 nm. 25 μL of the standard, sample or ethanol (blank) was incubated with 200 μL of DPPH, in a 96-wells plate, for 1 h, protected from the light. The absorbance was measured at 515 nm in a spectrophotometric microplate reader (Synergy H1 Hybrid Multi-mode Reader, BioTek, Winooski, Vermont, USA). The percentage of inhibition (%) was determined using the following equation:

$$\%\ inhibition = \left(1 - \frac{Absorbance\ of\ sample}{Absorbance\ of\ blank}\right) \times\ 100 \quad (1)$$

Trolox Equivalent Antioxidant Capacity (TEAC) was expressed as mM of Trolox equivalent (TE) per mL of nanoemulsion or free oil (mM TE/mL) using the following equations:

$$TEAC = concentration\ given\ by\ calibration\ curve\ \times \frac{volume\ of\ solvent\ used\ in\ the\ dilution}{volume\ of\ sample\ used} \quad (2)$$

2.7.1. ABTS assay

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) method was performed following Re et al. (1999), with modifications. In the day before the analysis, solutions of 7 mM ABTS and a solution of 2.45 mM potassium persulfate (PP), in Milli-Q water, were prepared. These

two solutions were then mixed at 1:1 ratio and left reacting for 12-16 h, under agitation and protected from light. In the day of the analysis, it was prepared a stock solution of 1000 μM Trolox in ethanol 60% and various Trolox standards for the calibration curve with concentrations between 700 and 15 μM. All the samples were diluted in ethanol 99%, with different proportions (1:20, 1:40 or 1:80) in order to fit the calibration curve. The ABTS:PP solution was dissolved in Milli-Q water to an absorbance value of 0.70 ± 0.02 at 734 nm. In a multi-plate it was put 10 μL of the standard, sample or Milli-Q water (blank) and 200 μL of ABTS:PP solution. These mixtures were left to rest for 6 minutes protected from the light and then their absorbance at 734 nm was measured. The percentage of inhibition and Trolox Equivalent Antioxidant Capacity were calculated using equations 2 and 3, respectively.

2.8. Cytotoxicity studies

The cytotoxicity of nanoemulsions was determined indirectly assessing the cellular viability by MTS or resazurin assays, measuring absorbance or fluorescence, respectively, after incubation with Caco-2 cells.

2.8.1. Cell culture

The cell viability assessment was performed with Caco-2 cells, clone HTB-37™, from human colon carcinoma, obtained from the American Type Culture Collection (ATCC®). Caco-2 cells (passage 26-40) were cultured in minimum essential medium (MEM), supplemented with 20% fetal bovine serum, 1% sodium pyruvate and 1% penicillin/streptomycin. The cells were kept at 37 °C and 5% CO_2 in 75 cm^2 flasks. For the *in vitro* assay, confluent cells were detached using 0.25% trypsin-EDTA solution, then precipitated by centrifugation at 1080 rpm for 5 min and resuspended in fresh medium at a concentration of 1×10^5 cells/mL. Cells were seeded onto 96-wells plates using of 1×10^4 cells (100 μL of cellular suspension) per well and left adhering overnight in a humidified atmosphere of 5% CO_2 in air at 37 °C.

After overnight cell adhesion, the culture medium was removed and replaced by 200 µL of samples (nanoemulsions with concentrations between 2 and 200 µg oil/mL emulsion) or controls and incubated for 24 or 48 h. A negative control was performed using the cells growing in the culture medium or 40% (v/v) DMSO was used as a positive control. The cell viability was expressed in percentage of absorbance/fluorescence in treated cells ($Signal_{TC}$) in relation to the absorbance/fluorescence of cells growing in the cell culture medium ($Signal_C$), as follows:

$$\%\ cell\ viability = \frac{Signal_{TC}}{Signal_C} \times 100 \quad (3)$$

2.8.2. MTS assay

At each time point, samples were removed and 100 µL of MTS 5% (v/v) in culture medium were added and incubated for 3h. The absorbance was read at 450 nm using a Microplate Reader (Synergy H1, BioTek, Winooski, Vermont, USA).

2.8.3. Resazurin assay

At each time point, samples were removed and 100 µL of resazurin 10% (v/v) in culture medium (0.01 mg/mL final concentration) were added and incubated for 3h. The fluorescence intensity was read at an excitation wavelength of 560 nm and an emission wavelength of 590 nm, using a Microplate Reader (Synergy H1, BioTek, Winooski, Vermont, USA).

2.9. Statistical analysis

All the tests were performed at least in triplicate and results were analysed by the statistical test one-way ANOVA, in the GraphPad Prism software (version 8.2.1, San Diego, California, USA) with a confidence interval of 95%.

3. Results and Discussion

3.1. Development of nanoemulsions

3.1.1. Influence of surfactant-to-oil ratio (SOR)

The mean size of nanoemulsions was around 200 nm for all tested conditions (SOR 1, 1.5 and 2): 196.0 ± 11.2 nm (SOR 1), 201.7 ± 6.7 nm (SOR 1.5) and 180.1 ± 1.6 nm (SOR 2). On the contrary, the polydispersity index (PDI) was highly affected by the change in SOR, decreasing for higher SOR values: 0.324 ± 0.058 (SOR 1), 0.176 ± 0.032 (SOR 1.5) and 0.130 ± 0.039 (SOR 2). Only samples with a SOR of 1.5 and 2 presented PDI values below 0.20. The intermediate o/w/o multiple emulsion, formed during the EPI method process, is crucial for the formation of small droplets in the final emulsion; these smaller droplets have a larger surface area, requiring higher quantities of surfactant to stabilise them. So, if there is not sufficient surfactant to cover all the interfacial areas of all the droplets formed, there is not an efficient decrease in the interfacial tension of such large areas and, consequently, some of the droplets would coalesce when colliding with each other, growing and increasing the PDI (Komaiko & McClements, 2015; Mayer et al., 2013; Ostertag et al., 2012). Using high surfactant-to-oil ratios, this can be avoided, which explains the obtained results, where higher concentrations of surfactant resulted in nanoemulsions with lower PDI values. Mayer *et al*. (2013) reported a different behaviour when evaluating the influence of SOR on PDI of nanoemulsions produced by the EPI method using Tween 80, as surfactant, with 10 wt% medium chain triglycerides (MCT) and Vitamin E Acetate, as lipid phase. They showed that the increase of SOR values led to an increase of the PDI values. In their study, a SOR of 1 and 2 produced nanoemulsions with sizes of 90 nm and 40 nm, respectively. Despite having small sizes, these formulations presented high PDI values for high SOR values, increasing from 0.21 (SOR 1) to 0.47 (SOR 2) (Mayer et al., 2013).

In 2016, Borrin *et al*. produced nanoemulsions using soybean oil and a SOR 1 and obtained sizes of 295 nm with a bimodal distribution (Borrin et al., 2016). In another study, formulations with MCT and a SOR 2 presented sizes of 100 nm, however the size increased to 600 nm when the MCTs were replaced by long chain triglycerides (LCT) and a high SOR (2.5) was used. Results show that the type of oil has a great influence on the nanoemulsions' size (Ostertag et al., 2012).

284 Generally, when MCTs are used, the nanoemulsions present a smaller mean size if compared with
285 LCTs. Despite this behaviour, the results obtained in this work showed that it is possible to achieve
286 nanoemulsions with good size and PDI values using a LCT (i.e. sunflower oil). The developed
287 nanoemulsions presented a mean size below 200 nm and a monomodal distribution. In addition, the
288 use of refined sunflower oil presented several advantages since it is 100% vegetal, low price, easy to
289 acquire and highly used within the food industry. In order to select the adequate surfactant-to-oil
290 ratio, it was defined that the mean diameters should be below 200 nm and the PDI should be below
291 0.20. Within these values an emulsion with a monodisperse size distribution, good homogeneity and
292 stability is expected (Guerra-Rosas et al., 2016; Komaiko & McClements, 2015; Yildirim, Oztop, &
293 Soyer, 2017). Thus, nanoemulsions formulated with a SOR 1.5 and 2 were selected for the
294 encapsulation of the essential oils.
295
296 *3.1.2. Encapsulation of essential oils*
297 The nanoemulsions characteristics (mean size and PDI) must be maintained after the
298 encapsulation process. Zeta potential is also an important parameter of stability and particles must
299 have a positive or negative zeta potential bigger than 30 mV, in order to achieve high repulsion forces,
300 leading to an electrostatic stabilisation (Gumustas, Sengel-Turk, Gumustas, Ozkan, & Uslu, 2017).
301 Different amounts of essential oils (from 0.5 to 2.0 wt%) were loaded into nanoemulsions
302 produced with SOR 1.5 and SOR 2 (**Table 1**). All samples were homogeneous and with a visual
303 aspect similar to formulations without EOs. The incorporation of EO in the nanoemulsions had a
304 slight influence (p>0.05) on PDI values in both SORs. In the case of SOR 1.5, the addition of EOs
305 led to a variation of zeta potential to more positive values (close to zero). The droplet size increased
306 with the incorporation of EOs in the case of SOR 2. Despite the statistically significant differences,
307 formulations with 0.5, 1 and 2 wt% of essential oil presented all the required parameters: PDI < 0.20,
308 mean size < 200 nm and zeta potential > ± 30 mV.

Nanoemulsions were tested with higher concentrations of essential oils (3 and 4 wt%) and SOR 2. Nanoemulsions with SOR 1.5 were not tested with high amounts of essential oils due to their instability during storage, as it will be presented in section 3.3. However, higher quantities of EO did not result in nanoemulsions with good PDI and mean size values, i.e. polydispersity below 0.20, and mean size below 200 nm, and therefore were not considered in further tests. These results are in agreement with a study using nanoemulsions produced by PIT method, with oregano essential oil, sunflower oil, Cremophor RH 40 and Span 80. Results showed an increase of PDI for nanoemulsions with higher amounts of essential oil in the formulation, ranging from 0.08 (5% essential oil) to 0.16 (7% essential oil) (Moraes-Lovison et al., 2017). This behaviour can be explained by the chemical structure of the essential oils that could affect their solubility in water. Nanoemulsions loaded with EOs have some solubility in water, affecting their stability and therefore the tendency to suffer Oswald ripening (Chang et al., 2015). Chang et al. (2012) produced nanoemulsions with pure thyme essential oil, as oil phase, and Tween 80, using a high-pressure homogeniser, a high energy method. They reported that the resulting emulsions had enormous sizes (1300 nm) and were highly unstable to droplet growth, creaming and oiling off, as a result of Ostwald ripening (Chang, McLandsborough, & McClements, 2012). It was demonstrated that the PDI and size of nanoemulsions tend to increase for higher concentrations of essential oil. However, there are some studies with low energy methods combined with MCT that showed a different behaviour. Lou et al. (2017) produced nanoemulsions using MCT with essential oil of *Citrus medica* and Tween 80 (SOR 2) by spontaneous emulsification (SE). They showed that the mean size decreases for higher concentrations of essential oil, being 165 nm for samples with 20% of essential oil in the lipid phase and 73 nm with 50%. Then, the mean size increased to 95 nm in formulations with 60% essential oil (Lou et al., 2017). Likewise, samples with MCT, orange oil and Tween 80 (SOR 2, SE method) had a decrease in droplet size with the increase of orange oil in the lipid phase up to 50%, and then for higher concentrations it suffered a considerable increase (Chang & McClements, 2014). The same behavior was presented by Chang et al. (2013), that showed a decrease of the mean size of nanoemulsions with MCT and Tween 80 (SOR 1, SE

335 method) from 160 nm to 60 nm when the oil phase changed to 25% of carvacrol oil and 75% MCT.
336 Results also showed a size increased to 800 nm when the concentration of carvacrol reached 60%
337 (Chang, McLandsborough, & McClements, 2013). None of these works referred PDI and therefore
338 is not clear if the mean sizes presented are resultant from a monomodal or bimodal distribution. A
339 study on nanoemulsions with cinnamon oil, MCT and Tween 80 (SOR 2), produced by the PIT
340 method, showed that intermediate concentrations of essential oil (30- 40 % in the oil phase) lead to
341 lower PDI (0.17) and sizes (100-107 nm), being larger sizes obtained at lower (0-20 %) and higher
342 (60-100 %) concentrations, with broad multimodal size distributions (Chuesiang et al., 2018). The
343 same happened in formulations with cinnamon oil, coconut oil (MCT) and Tween 80 (SOR 1),
344 produced by the SE method (Yildirim et al., 2017). In another study using a low energy method, a
345 nanoemulsion with 4 wt% oil phase composed by 100% D-limonene and a SOR of 1.5 presented
346 sizes of 40 nm, with a bimodal distribution and, when the oil phase was changed to 85 % D-limonene
347 and 15 % of sunflower oil, the droplet size increased to 120 nm, maintaining a bimodal size
348 distribution. (Li et al., 2013).
349
350 *3.2. Transmission Electron Microscopy (TEM) morphologic analysis*
351 The morphological analysis of nanoemulsions with TEM allowed the confirmation that all
352 formulations, with and without essential oil, have a spherical shape. **Figure 1** presents the TEM
353 analysis of a NE using a SOR of 2. The core-shell structure of the particles is visible and the shell
354 layer of Tween 80 is establishing the oil-water interface.
355
356 *3.3. Stability of nanoemulsions during storage*
357 The stability of nanoemulsions was evaluated in terms of size distribution, PDI, zeta potential and
358 creaming, during storage at 4 and 25 °C (**Figures S1** and **S2**). The values of zeta potential (results
359 not shown) ranged between - 53 mV and -35 mV during storage. Formulations with only sunflower
360 oil (NE_1.5 and NE_2) showed to be stable for 6 months when stored at 4 °C. Nanoemulsions loaded

with EOs and with a SOR of 1.5 were monitored only for one month, since at the third day of storage they showed destabilisation through the appearance of a great creaming layer. This was mainly observed for samples loaded with lemon balm oil (NE_1.5_LB0.5, NE_1.5_LB1 and NE_1.5_LB2) and coincided with a significant reduction in size and PDI variations. This creaming could have occurred since the larger droplets tend to migrate fast to the top (creaming) (Silva et al., 2015). Also, some coalescence could have occurred, contributing to the appearance of creaming. Thus, the reduction of the mean size can be explained by the migration of the larger droplets, leaving the smaller in the nanoemulsion and showing that the two phenomena are related (creaming and smaller sizes). This migration of droplets usually occurs some hours after the emulsification (Guerra-Rosas et al., 2016), explaining why it was noticed on the third day of storage. Additionally, nanoemulsions loaded with essential oils have the tendency to suffer Oswald ripening due to the solubility of EOs in water (Chang et al., 2015).

Formulations of SOR 2 loaded with essential oils did not show evident creaming and were stable for three months (Figure S2), mainly when refrigerated and regardless of the amount of essential oil loaded. The results are in agreement with Chang et al. (2012), that used a high energy method (high-pressure homogenisation). They showed that nanoemulsions with 3 wt% thyme essential oil, 7 wt % corn oil and Tween 80 (SOR 0.1), were stable during 30 days at 20 °C, without changes in particle size and PDI (Chang et al., 2012). In the present work, it can be said that the results were comparable to the ones obtained by high energy methods once the PDI and size remained stable, even during 3 months.

In another study, nanoemulsions with D-limonene and Tween 80 (SOR 1.5, EPI method) showed an increase of the size when stored during 12 days. The initial droplet size was 40 nm, changing to 169 nm and108 nm, when stored at 4 °C and 28 °C, respectively (Li et al., 2013). Formulations with soybean oil and Tween 80 (SOR 1.0, EPI method), also increased droplet size after 15 days of storage, and a significant change in the distribution curve occurred, increasing its PDI (Borrin et al., 2016). Another study, showed that the mean size of nanoemulsions loaded with essential oil of *Ocimum*

387 *basilicum* and Tween 80 (SOR 1, EPI method) increased during 30 days of storage and their PDI
388 decreased but remaining a polymodal distribution (Sundararajan, Moola, Vivek, & Kumari, 2018).
389 Due to its high stability, nanoemulsions with a SOR of 2 were selected for the following tests.
390
391 *3.4. Antimicrobial activity of nanoemulsions*
392 **Table 2** shows the results for the antimicrobial activity of nanoemulsions with thyme or lemon
393 balm oil against *E. coli* and *S. aureus*.
394 Neat nanoemulsions (NE_2) inhibited *E. coli*, showing that the materials used for the
395 nanoemulsions production had antimicrobial activity against this bacterium. This was already
396 reported elsewhere for formulations using Tween 80 and oleic acid from sunflower oil (Nielsen,
397 Kjems, Mygind, Snabe, & Meyer, 2016; Yoon, Jackman, Valle-González, & Cho, 2018). However,
398 nanoemulsions with increasing concentrations of EOs did not induce higher antimicrobial activity
399 against *E. coli* (p>0.05). It was expected an effect of the essential oils once they are reported to possess
400 activity against this bacterium (Khorshidian, Yousefi, Khanniri, & Mortazavian, 2017; Nabavi et al.,
401 2015). However, is also known that Gram-negative bacteria, such as *E. coli*, are less susceptible to
402 essential oils than Gram-positive bacteria (Khorshidian et al., 2017; Pandey et al., 2017). Thus, it can
403 be suggested that the concentrations of thyme and lemon balm oils used were not sufficient to increase
404 the inhibition of *E. coli*.
405 *S. aureus* was only inhibited by nanoemulsions loaded with thyme and lemon balm oils, showing
406 that the inhibition of this bacterium was due exclusively to essential oils. Lou *et al*. (2017) tested the
407 antimicrobial activity of nanoemulsions loaded *with Citrus medica* essential oil (carrier oil: MCT;
408 surfactant: tween 80; SOR: 2; method: SE) against *E. coli* and *S. aureus*. Their formulations possessed
409 activity against both bacteria but presenting a greater influence on *S. aureus* (Lou et al., 2017). In this
410 work, despite not affecting *E. coli*, nanoemulsions with essential thyme and lemon balm oils reduced
411 the growth of the bacterium that they were most likely to inhibit, i.e. *S. aureus*. However, contrary to
412 the work of Lou *et al*. (2017), the inhibition of *S. aureus* did not show statistical differences (p>0.05)

413 for higher amounts of both essential oils. With this in mind, it could be said that formulations with
414 the lowest concentration of both oils can be used if the goal is the microbial inhibition.
415
416 *3.5. Antioxidant activity of nanoemulsions*
417 The antioxidant activity of the selected nanoemulsions was assessed using two different
418 methodologies, the DPPH free radical scavenging and the ABTS tests. The results obtained are
419 presented in **Figure 2.** Nanoemulsions loaded with increasing concentrations of lemon balm oil
420 showed no significant difference (p>0.05) compared to neat formulations (NE_2). On the other hand,
421 thyme essential oil, encapsulated or free, revealed antioxidant capacity and higher antioxidant activity
422 was observed for increasing concentrations of encapsulated essential oil.
423 Some studies showed that the DPPH radical scavenging activity of essential oils from
424 *Cymbopogon densiflorus*, *Citrus medica* and *Ocimum basilicum* was higher when they were
425 encapsulated into nanoemulsions produced by two low energy methods, the EPI and SE methods
426 (Lou et al., 2017; Seibert et al., 2019; Sundararajan et al., 2018). The nanoencapsulated *Cymbopogon*
427 *densiflorus* essential oil had also higher ABTS radical scavenging activity than the free counterpart
428 (Seibert et al., 2019). However, in the present work, the antioxidant activity was high for free thyme
429 essential oil.
430 The ABTS test provided higher TEAC values when compared to the DPPH method. The same
431 was observed by Seibert *et al*. (2019) who showed that inhibition results were higher for the ABTS
432 assay than for the DPPH test, justified by the higher sensitivity of ABTS test. They referred that
433 ABTS is more versatile since its working solution is soluble in both aqueous and organic solvents,
434 being able to evaluate the activity in polar and non-polar samples (Seibert et al., 2019). Despite the
435 different values obtained, both methods showed the same tendency.
436 Thyme essential oil showed to have the strongest antioxidant activity that can be explained by its
437 chemical composition. A previous study (not published results) about the composition of the two EOs
438 used in this work, showed that lemon balm essential oil was mainly composed by β-caryophyllene

and thyme essential oil had as main compounds thymol, *p*-cymene and γ-terpinene. Thus, thyme EO was rich in phenols that are known to have significant antioxidant activity and which explain this higher antioxidant capacity. NEs loaded with thyme EOs presented the higher antioxidant activity and simultaneous good antimicrobial activity and thus are the most promising loaded nanoemulsions for future applications. Therefore they were tested regarding their potential cytotoxicity.

3.6. Cytotoxicity studies

The cytotoxicity of nanoemulsions was indirectly evaluated by two different methods (MTS and resazurin) assessing cell viability after incubation (24 or 48h) with nanoemulsions. The results presented in **Figure 3** show that the viability of Caco-2 cells is reduced when incubated with higher concentrations of nanoemulsions and for longer periods. The same tendency was verified for both methodologies.

The results were expressed as a percentage of cell viability and as the oil concentration in the sample was increased, the viability of Caco-2 cells decreased. Therefore, the results can be presented in terms of IC50 that corresponds to the concentration at which cell viability is less than 50%. At 24 h of exposure, the formulation loaded with thyme essential oil (NE_2_T2) did not reach the IC50 with any of the tested concentrations. Nanoemulsions loaded with lemon balm essential oil (NE_2_LB2) presented cytotoxicity at approximately 120 µg oil/mL of NE and the neat formulation was toxic only at 190 µg oil/mL of NE.

According to MTS results, at 48 h of exposure, nanoemulsions with lemon balm oil showed an IC50 of approximately 100 µg of oil/mL of emulsion. Neat nanoemulsions had an IC50 of 120 µg/mL of emulsion and formulations with thyme oil present an IC50 of 140 µg/mL of emulsion. Thus, at both time points, lemon balm essential oil showed higher cytotoxicity than thyme oil.

Sousa et al. (2004) tested the cytotoxicity of lemon balm essential oil through the MTT assay at 48 h demonstrating that this oil was toxic at concentration ranged between 90 and 100 µg/mL, which is in good agreement to the results presented in this work (Sousa et al., 2004). Another study evaluated

the toxicity of thymol, one of the main compounds of thyme essential oil, and their results revealed that thymol was toxic to Caco-2 cells at concentrations higher than 100 μg/mL in a lactate dehydrogenase assay at 24 h of exposure (Putaala, Nurminen, & Tiihonen, 2017).

4. Conclusion

Nanoemulsions with surfactant-oil-ratio (SOR) of 1.5 and 2 were produced by the emulsion phase inversion method, using Tween 80 and sunflower oil. These formulations were loaded with 0.5, 1.0 and 2.0 wt% of thyme or lemon balm essential oils and maintained the intended characteristics, such as a size average below 200 nm and a polydispersity index lower than 0.2. Higher amounts of essential oil destabilised the system and were not able to produce stable nanoemulsions. In the stability test, samples with SOR 2 revealed to be more stable (up to 6 months without EO and 3 months with EO) when stored at 4 °C. DPPH and ABTS antioxidant tests showed that nanoemulsions loaded with thyme oil possessed higher antioxidant activity when compared with lemon balm. These nanoemulsions were not effective against *E. coli* but could inhibit *S. aureus* and this activity was only due to essential oils, since the nanoemulsion without EO did not inhibit this bacterium. Neat nanoemulsions (without EOs) revealed to be toxic in Caco-2 cells for 190 μg/mL of NE at 24 h and 120 μg/mL of NE at 48 h. Nanoemulsions loaded with thyme oil only showed cytotoxicity at 48 h of exposure at 140 μg/mL of NE. Formulations with lemon balm oil showed higher toxicity since they were toxic from 120 μg/mL of NE at 24 h and 100 μg/mL of NE at 48 h. In conclusion, stable nanoemulsions produced by a low energy method are able to encapsulate essential oils up to 20% $w/w_{oil\ phase}$. These nanoemulsions showed important biological activities (antimicrobial and antioxidant activity) and can be used up to a concentration of 100 μg oil/mL of NE without showing cytotoxicity.

CRediT authorship contribution statement

Cátia I. Sampaio: Conceptualisation, Methodology, Investigation, Writing - Original draft preparation, Writing - Review & Editing; Ana I. Bourbon: Conceptualisation, Methodology, Investigation, Writing - Review & Editing; Catarina Gonçalves: Methodology, Investigation, Writing - Review & Editing; Lorenzo: Writing - Review & Editing; Alice Dias: Writing - Review & Editing, Supervision; Miguel A. Cerqueira: Conceptualisation; Writing - Review & Editing, Supervision

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Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found online at **XXX**

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